Mutational Analysis of the Ethylene Receptor ETR1. Role of the Histidine Kinase Domain in Dominant Ethylene Insensitivity[1]

Rebekah L. Gamble, Xiang Qu, and G. Eric Schaller*

Department of Biochemistry and Molecular Biology, University of New Hampshire, Durham, New Hampshire 03824

The ethylene receptor family of Arabidopsis consists of five members, one of these being ETR1. The N-terminal half of ETR1 contains a hydrophobic domain responsible for ethylene binding and membrane localization. The C-terminal half of the polypeptide contains domains with homology to histidine (His) kinases and response regulators, signaling motifs originally identified in bacteria. The role of the His kinase domain in ethylene signaling was examined in planta. For this purpose, site-directed mutations were introduced into the full-length wild-type *ETR1* gene and into *etr1-1*, a mutant allele that confers dominant ethylene insensitivity on plants. The mutant forms of the receptor were expressed in Arabidopsis and the transgenic plants characterized for their ethylene responses. A mutation that eliminated His kinase activity did not affect the ability of etr1-1 to confer ethylene insensitivity. A truncated version of etr1-1 that lacks the His kinase domain also conferred ethylene insensitivity. Possible mechanisms by which a truncated version of etr1-1 could exert dominance are discussed.

The simple gas ethylene functions as an endogenous regulator of plant growth and development (Abeles et al., 1992). Ethylene regulates seed germination, seedling growth, leaf and petal abscission, fruit ripening, organ senescence, and pathogen responses. Ethylene perception in Arabidopsis is mediated by a family of five receptors: ETR1, ERS1, ETR2, ERS2, and EIN4 (Bleecker, 1999; Chang and Shockey, 1999). Of these receptors, ETR1 has been characterized in most detail because it was the first member of the receptor family identified (Chang et al., 1993; Schaller and Bleecker, 1995).

The N-terminal half of ETR1 is involved in signal input. This region of ETR1 contains three predicted transmembrane segments that encompass the ethylene-binding site (Schaller and Bleecker, 1995). A copper cofactor is a necessary part of the ethylene-binding site, presumably serving to ligand the ethylene (Rodriguez et al., 1999). ETR1 forms a disulfide-linked dimer in the membrane, with dimerization mediated by two cysteines located near the N terminus (Schaller et al., 1995). Following the transmembrane segments, ETR1 contains a GAF domain; GAF domains, initially identified in cGMP-specific and -stimulated phosphodiesterases, adenylate cyclases, and the *Escherichia coli* protein FhlA, are involved in cGMP binding and light regulation, but their function in ETR1 is unknown (Aravind and Ponting, 1997).

The C-terminal half of ETR1 is likely to be involved in signal output. This portion of the protein contains regions with homology to His kinases and the receiver domains of response regulators (Chang et al., 1993). These represent signaling elements originally identified in bacterial signal transduction systems (Parkinson, 1993), but which are now known to be present in plants and fungi as well (Schaller, 2000). In many of these signal transduction systems, the His kinase domain autophosphorylates at a conserved His residue in response to an environmental stimulus. This phosphate is then transferred to a conserved Asp residue within the receiver domain of the response regulator. Phosphorylation of the response regulator modulates its ability to mediate downstream signaling in the pathway. Some bacterial His kinases also contain a phosphatase activity that will dephosphorylate the response regulator. His kinase activity has been demonstrated for ETR1 (Gamble et al., 1998), but the role of this activity in ethylene signal transduction has not been determined. In addition, ETR1 has been shown to interact through both its His kinase and receiver domains with CTR1 (Clark et al., 1998), a downstream element of the ethylene signal transduction pathway (Kieber et al., 1993). CTR1 is related to the Raf-type Ser/Thr protein kinases from mammals, indicating that ethylene signal transduction could feed into a MAP kinase cascade, with CTR1 representing a MAPKKK (Kieber et al., 1993). ETR1 could potentially regulate activity of CTR1 through enzymatic or allosteric mechanisms.

[1] This work was supported by the National Science Foundation (grant nos. MCB–9603679 and MCB–9982510 to G.E.S.). This is scientific contribution no. 2,107 from the New Hampshire Agricultural Experiment Station.

* Corresponding author; e-mail egs@cisunix.unh.edu; fax 603–862–4013.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010777.

The other four members of the Arabidopsis ethylene receptor family are similar in overall structure to ETR1, with the greatest level of amino acid conservation being found in the ethylene-binding domains (Chang and Shockey, 1999). However, some differences among family members are notable. In particular, ETR2, ERS2, and EIN4 contain diverged His kinase domains and lack residues considered essential for His kinase activity (Chang and Shockey, 1999). Two of the proteins (ERS1 and ERS2) lack a receiver domain at the C terminus (Chang and Shockey, 1999). To directly assess the role of the ethylene receptor family in ethylene perception, loss-of-function mutations have been isolated in four of the five gene members of the family (Hua and Meyerowitz, 1998). Single loss-of-function mutations have little or no effect upon ethylene signal transduction. However, in combination, the mutants show constitutive ethylene responses. This effect is most pronounced in triple and quadruple loss-of-function mutations (Hua and Meyerowitz, 1998). These results indicate that there is functional redundancy among the receptor family members. In addition, because elimination of receptors activates ethylene responses, these results support a model in which the receptors repress the ethylene responses in the absence of ethylene. According to this model, binding of ethylene inactivates receptor signaling, thereby relieving the repression on the ethylene pathway.

Dominant ethylene-insensitive mutations of the receptors have been identified that apparently lock the receptor into a signaling state such that it represses ethylene responses whether the plants are grown in the presence or absence of ethylene. Dominant ethylene insensitivity can be conferred by mutations in the receptor that disrupt ethylene binding or that uncouple ethylene binding from signal output (Hall et al., 1999). The dominant *etr1-1* mutation arises from the change of a single amino acid (Cys65Tyr) and has been shown to eliminate binding of the copper cofactor and consequently prevents ethylene binding to the receptor (Schaller and Bleecker, 1995; Rodriguez et al., 1999). A mutation within the ethylene-binding site of any one of the five receptor isoforms can result in dominant ethylene insensitivity (Chang et al., 1993; Hua et al., 1995, 1998; Sakai et al., 1998). The effect of dominant ethylene-insensitive mutations is not limited to Arabidopsis (Wilkinson et al., 1997). The Arabidopsis *etr1-1* mutant can confer dominant ethylene insensitivity in transgenic tomato (*Lycopersicon esculentum*; Wilkinson et al., 1997), a genetic background in which tomato would be expressing its own family of at least five ethylene receptors (Tieman and Klee, 1999). Similarly, introduction of mutant tomato ethylene receptors into Arabidopsis also confers dominant ethylene insensitivity (Tieman and Klee, 1999). In this study, we used the mutant receptor etr1-1 as a tool to examine ethylene signal transduction in Arabidopsis, studies facilitated by the dominant nature of this mutation. We focused on the role that the His kinase domain plays in mediating the effects of the etr1-1 mutant receptor. Our results lend insight into the mechanism of dominance of the *etr1-1* mutation and also into the general mechanism of ethylene signal transduction.

RESULTS

A G2 Box Mutation Eliminates His Kinase Activity of the Ethylene Receptor ETR1

The ETR1 protein has a modular structure, with His kinase and receiver domains located in the C-terminal half of the protein (Fig. 1A). The His kinase domain contains conserved residues considered essential for enzymatic activity based on the well-characterized His kinases of bacteria (Stock et al., 1995). These include a His residue that serves as the presumptive site of autophosphorylation and a catalytic domain with two regions of conserved Gly residues referred to as the G1 and G2 boxes. To

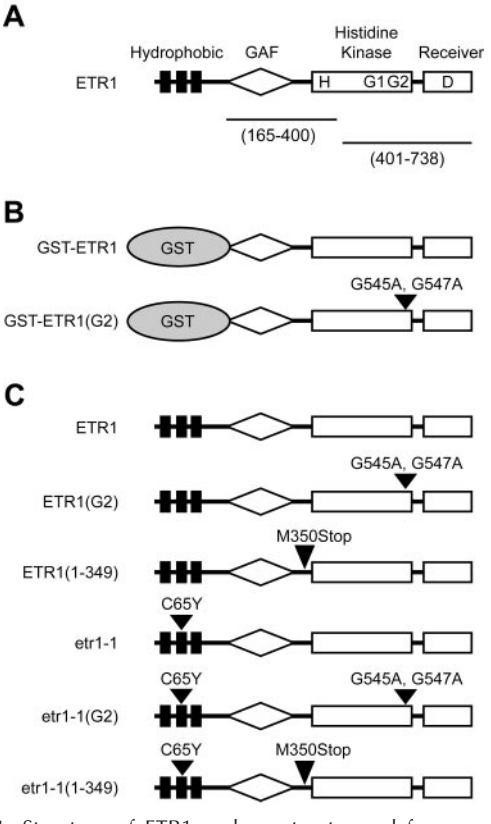

Figure 1. Structure of ETR1 and constructs used for experimental analysis. The hydrophobic ethylene-sensing domain, the GAF domain, the His kinase domain, and the receiver domain are indicated. H indicates His-353 and D indicates Asp-659, the putative phosphorylation sites. G1 and G2 indicate positions of the G1 and G2 boxes within the kinase domain. A, Domains of the full-length ETR1 protein. Positions of regions used to generate the anti-ETR1(165–400) and anti-ETR1(401–738) antibodies are indicated. B, Versions of ETR1 expressed as glutathione *S*-transferase (GST) fusions in yeast. C, Versions of ETR1 expressed as transgenes in Arabidopsis.

analyze His kinase activity of ETR1, we transgenically expressed soluble portions of the protein as fusions with GST (Fig. 1B) in yeast (*Saccharomyces cerevisiae*; Gamble et al., 1998). As shown in Figure 2, the purified GST-ETR1 fusion exhibits autophosphorylation activity. The incorporated phosphate is resistant to alkali treatment, but is sensitive to acid treatment, consistent with phosphorylation of a His residue (Fig. 2; Duclos et al., 1991). We have shown previously that autophosphorylation can be abolished by mutations that eliminate either the presumptive site of autophosphorylation (His-353) or residues within the G1 box of the catalytic domain of ETR1 (Gamble et al., 1998). Mutation of the G2 box is also predicted to abolish His kinase activity because it contains conserved residues implicated in ATP binding (Bilwes et al., 1998). We observed no phosphorylation in a GST-ETR1 fusion containing a mutated G2 box (G545A and G547A), demonstrating the necessity of the G2 box for His kinase activity (Fig. 2). Western-blot analysis was performed to confirm equivalent protein loading.

Effect of a G2 Box Mutation in the Ethylene Receptor on the Seedling Growth Response

To study the in vivo effects of mutations in the ETR1 ethylene receptor, we took advantage of the ethylene-induced "triple response" in seedlings (Knight et al., 1910). Ethylene has a pronounced effect upon wild-type seedlings grown in the dark. As shown in Figure 3A, the triple response of Arabidopsis seedlings to ethylene is characterized by an inhibition of hypocotyl and root elongation, an exaggerated apical hook, and a thickening of the hypocotyl (Bleecker et al., 1988; Guzmán and Ecker, 1990). The *etr1-1* ethylene-insensitive mutant of Arabidopsis lacks the triple response and instead has the elongated hypocotyl and characteristic etiolated morphology of an air-grown seedling (Fig. 3A; Bleecker et al., 1988).

To examine the function of *ETR1* and *etr1-1* as transgenes in planta, 7.3-kb genomic fragments containing promoter and coding regions were cloned into plant transformation vectors and used to transform Arabidopsis. Transgenic seedlings were initially identified on the basis of kanamycin resistance (kan^r), and the subsequent generation was scored for

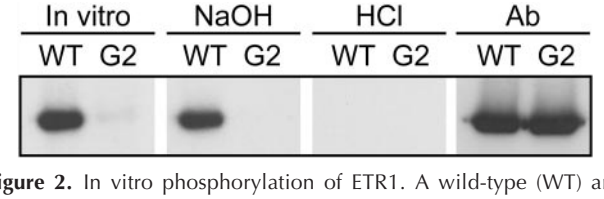

Figure 2. In vitro phosphorylation of ETR1. A wild-type (WT) and G2-box mutant version (G2) of the fusion-protein GST-ETR1 were examined for the ability to autophosphorylate. Affinity-purified proteins were incubated with γ^{32}P-ATP, subjected to SDS-PAGE, then transferred to nylon membrane (in vitro). Proteins were then sequentially treated with alkali (NaOH) and acid (HCl). Incorporated phosphate was visualized after each treatment by autoradiography. Finally, protein was visualized by western blot using the anti-ETR1(401–738) antibody.

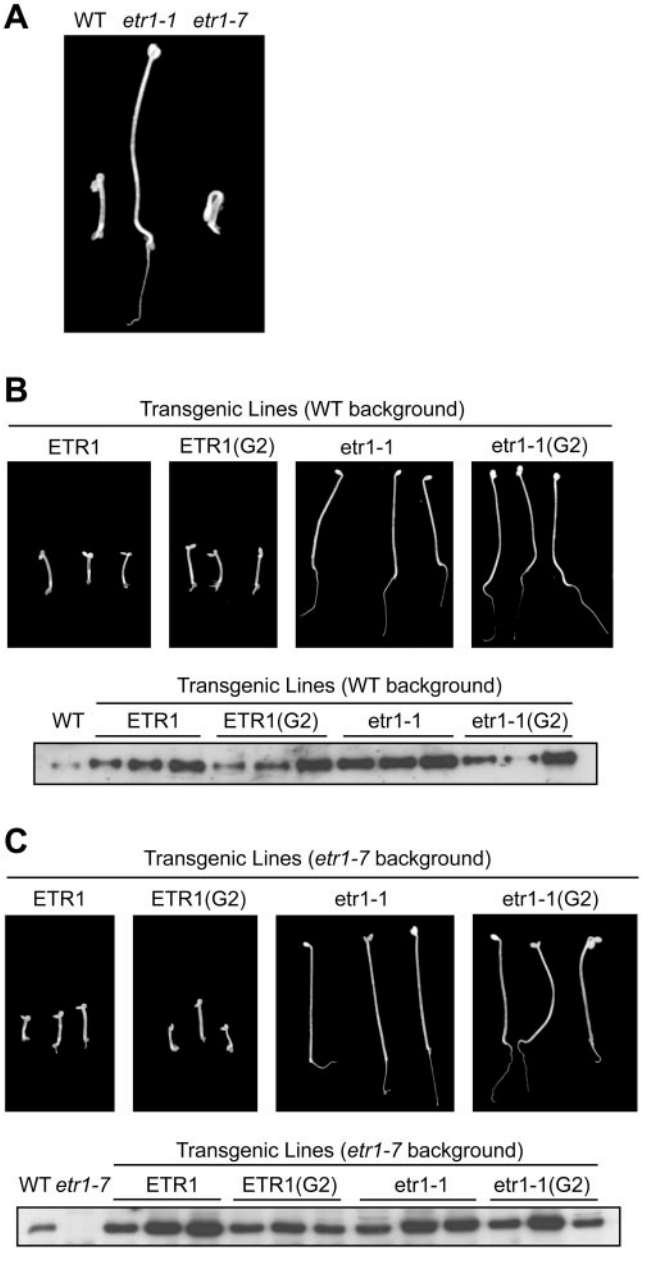

Figure 3. Effect of mutant ETR1 receptors upon the triple response of dark-grown seedlings. Phenotypes of 4-d-old seedlings grown in 35 μL L^{-1} ethylene are shown. Representative seedlings from three independent lines for each transgene are shown. The level of immunodetectable full-length receptor for each of the plant lines was determined by western-blot analysis using the anti-ETR1(401–738) antibody. A, Response of wild type (WT), the ethylene-insensitive mutant *etr1-1*, and the loss-of-function mutant *etr1-7* to ethylene. B, Ethylene response of the wild-type Arabidopsis transformed with *ETR1*, *ETR1(G2)*, *etr1-1*, and *etr1-1(G2)*. C, Ethylene response of the loss-of-function *etr1-7* line transformed with *ETR1*, *ETR1(G2)*, *etr1-1*, and *etr1-1(G2)*.

ethylene sensitivity based on the triple response. Transformation of wild-type plants with the *etr1-1* genomic clone yielded ethylene-insensitive plants with high frequency (Table I; Fig. 3B). In contrast, all of the plants transformed with a wild-type ETR1 genomic fragment displayed the normal triple response to ethylene (Table I; Fig. 3B). The expression level of ETR1 protein in transgenic plants was determined by performing western-blot analysis on membranes isolated from etiolated seedlings (Fig. 3B). Both the *ETR1* and *etr1-1* transgenic lines had higher levels of the immunodetectable protein when compared with the level in wild-type seedlings; this is consistent with expression of the transgene. The amount of immunodetectable protein was similar in both the *ETR1* and *etr1-1* transgenic lines; therefore, an increased level of expression cannot account for the ethylene insensitivity observed in the *etr1-1* transgenic lines.

The wild-type and *etr1-1* genomic fragments were mutated to eliminate residues of the G2 box (Fig. 1C). The G2 box was chosen for mutation because it eliminates His kinase activity of ETR1 (Fig. 2). In addition, mutation of the G2 box should eliminate any potential phosphatase activity of the protein (Yang and Inouye, 1993), an additional enzymatic activity found in some bacterial His kinases. Wild-type plants transformed with *etr1-1(G2)* yielded ethylene-insensitive seedlings with high frequency, but all wild-type plants transformed with *ETR1(G2)* displayed the triple response to ethylene (Table I; Fig. 3B). Immunodetectable ETR1 protein in the *ETR1(G2)* and *etr1-1(G2)* transgenic lines demonstrated similar variability in their expression levels, and were above the level of ETR1 in wild-type seedlings. Thus, the ethylene insensitive phenotype produced by *etr1-1* does not require enzymatic activity of the His kinase domain. Ethylene insensitivity is dominant because the genetic background contains wild-type ETR1.

To examine the effect of the transgenes in a background that lacked ETR1, we transformed the same constructs into the *etr1-7* genetic background. The *etr1-7* mutant is a loss-of-function allele of *ETR1* that arises from a stop codon at Trp74 (Hua and Meyerowitz, 1998). Dark-grown seedlings of *etr1-7* are responsive to ethylene (Fig. 3A) and do not make any immunodetectable ETR1 protein (Fig. 3C). The ethylene responsiveness of the *etr1-7* mutant is mediated by the remaining four members of the ethylene receptor family. Results from the expression of transgenes in the *etr1-7* background were similar to those obtained with the wild-type background (Table I; Fig. 3). Both *etr1-1* and *etr1-1(G2)* conferred ethylene insensitivity upon transgenic *etr1-7* seedlings. Control lines transformed with *ETR1* or *ETR1(G2)* were responsive to ethylene. The levels of immunodetectable ETR1 protein correspond to expression from the transgene because of the absence of endogenous ETR1 protein (Fig. 3C). Thus, kinase activity is not required for the ethylene insensitivity conferred by etr1-1 in a genetic background that lacks wild-type ETR1.

Effect of a Truncated Receptor on the Seedling Growth Response

To further assess the role of the C-terminal half of ETR1 in ethylene responses, we generated truncated versions of the receptor that lacked the His kinase and receiver domains. Truncated versions of the receptor were coded for in the 7.3-kb genomic fragments by mutation of Met350 to a stop codon (Fig. 1). Although nonsense-mediated decay of mRNAs containing premature stop codons has been demonstrated to occur in plants (van Hoof and Green, 1996), we have observed that message is still produced from the *ETR1* loss-of-function mutations that contain premature stop codons (X. Qu and G.E. Schaller, unpublished data). Thus, by the introduction of stop

Table I. *Ethylene sensitivity of transgenic lines*

Transgenic lines were initially identified on the basis of kan^r. Seeds from the subsequent generation of each line were then screened for the presence of ethylene-insensitive seedlings using the triple-response assay.

Transgene	Background	kan^r Lines	Ethylene-Insensitive Lines	Ethylene-Insensitive Lines
				%
ETR1	WT	10	0	0
ETR1(G2)	WT	12	0	0
ETR1(1–349)	WT	16	0	0
etr1-1	WT	14	13	93
etr1-1(G2)	WT	10	10	100
etr1-1(1–349)	WT	12	10	83
ETR1	*etr1-7*	12	0	0
ETR1(G2)	*etr1-7*	13	0	0
ETR1(1–349)	*etr1-7*	22	0	0
etr1-1	*etr1-7*	17	13	76
etr1-1(G2)	*etr1-7*	20	15	75
etr1-1(1–349)	*etr1-7*	16	7	44

codons, truncated versions of ETR1 can theoretically be produced that preserve downstream non-coding determinants of expression. Mutant versions of the receptor were cloned into a plant transformation vector and used to transform Arabidopsis.

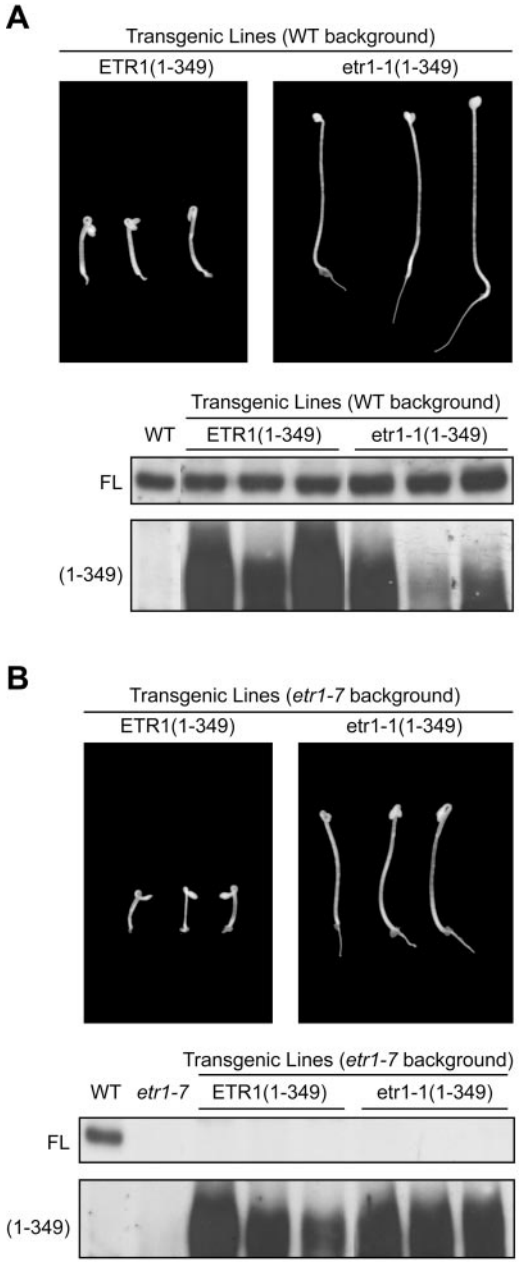

Figure 4. Effect of the ETR1(1–349) and etr1-1(1–349) mutant receptors upon the triple response. The *ETR1(1–349)* and *etr1-1(1–349)* transgenes were transformed into either wild-type Arabidopsis (A) or into the loss-of-function *etr1-7* line (B). Representative seedlings from three independent lines for each transgene are shown after 4 d growth in 35 μL L^{-1} ethylene. The level of immunodetectable full-length receptor (FL) for each of the plant lines was determined by western-blot analysis using the anti-ETR1(401–738) antibody with 10 μg protein. The level of immunodetectable protein for the truncated receptors (1–349) was determined using the anti-ETR1(165–400) antibody.

As shown in Table I and Figure 4A, wild-type plants transformed with *etr1-1(1–349)* yielded ethylene-insensitive seedlings at high frequency. In contrast, all wild-type plants transformed with the control *ETR1(1–349)* were sensitive to ethylene. An immunodetectable protein was recognized by an antibody generated against amino acids 165 through 400 of ETR1 (Fig. 4A) in the transgenic lines at a molecular mass consistent with that of a truncated receptor. As expected, we still observed the full-length ETR1 receptor endogenous to the wild-type line into which the transgenes were transformed (Fig. 4A). The levels of full-length ETR1 detected in the transgenic lines were similar to those found in the control wild-type line, indicating that expression of the truncated receptor did not affect expression of the native full-length receptor. Based on these results, we concluded that a truncated version of the etr1-1 mutant receptor lacking the His kinase and receiver domains was still capable of conferring dominant ethylene insensitivity upon wild-type plants.

ETR1(1–349) and *etr1-1(1–349)* were also transformed into the *etr1-7* genetic background of Arabidopsis so as to observe their effects on a plant that lacks ETR1. Ethylene insensitivity was observed in plants transformed with *etr1-1(1–349)*, but not in plants transformed with the control *ETR1(1–349)* (Table I; Fig. 4B). Western-blot analysis confirmed that the transgenic *etr1-7* lines lacked the full-length ETR1 receptor but expressed a truncated version of the receptor.

Quantitative Analysis of the Seedling Growth Response to Ethylene

To gain more information about the ethylene insensitivity conferred by the transgenes, we performed a quantitative analysis. We primarily focused on the transgenic *etr1-7* lines because, lacking the native ETR1 receptor, the level of protein expression from the transgene can be immunologically determined. Quantitative analysis of ethylene responses was performed with transgenic lines that segregated for kan^r as single loci, using homozygous seed obtained from plants allowed to self-pollinate. For each transgene, two independent transgenic lines were characterized that had been scored as ethylene insensitive based on their lack of a triple-response phenotype. Seedlings were grown in the dark in ethylene concentrations ranging from 0 to 1,000 μL L^{-1}, and hypocotyl lengths measured after 4 d growth. Transgenic lines containing different versions of *etr1-1* were compared with control untransformed lines.

As shown in Figure 5, A through D, two independent transgenic *etr1-7* lines containing the *etr1-1* and *etr1-1(G2)* transgenes showed no responsiveness to ethylene even at the highest ethylene concentration tested (1,000 μL L^{-1}). In contrast, two independent transgenic *etr1-7* lines containing the *etr1-1(1–349)*

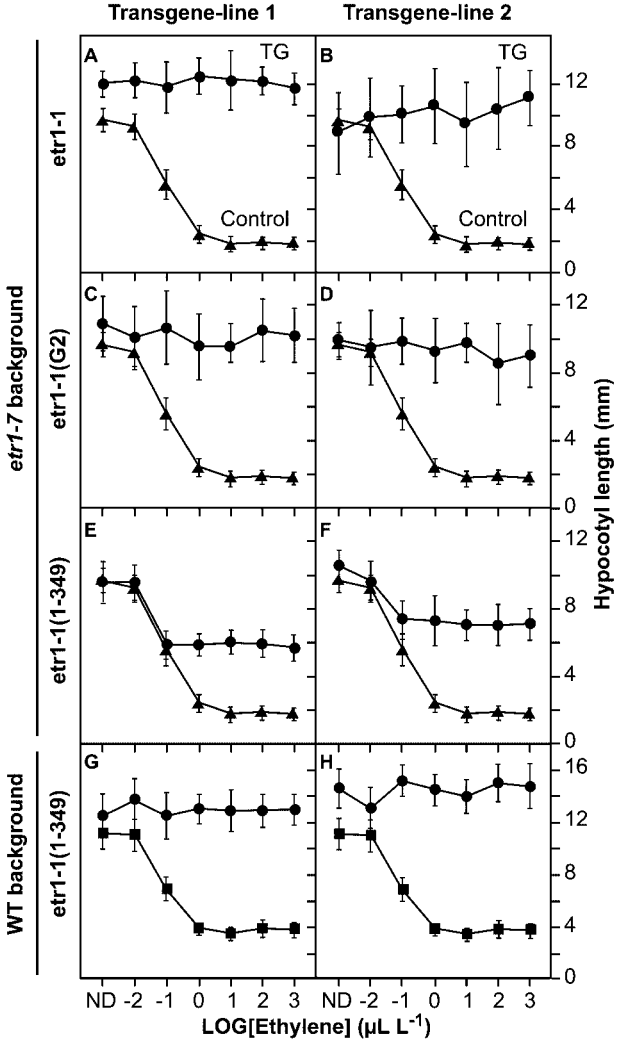

Figure 5. Ethylene dose response curves of hypocotyl growth for *etr1-1* mutants. Dose response curves from two independent transgenic lines (black circles, TG) for each transgene are shown. Results are shown for *etr1-1* (A and B), *etr1-1(G2)* (C and D), and *etr1-1(1–349)* (E and F) in the *etr1-7* background. Results are also shown for *etr1-1(1–349)* in a wild-type background (G and H). For comparison, ethylene dose response curves are shown for control wild-type (black square) and *etr1-7* (black triangle) hypocotyls. Values represent the means ± SD of 25 measurements. ND, No detectable ethylene.

transgene did show some responsiveness to ethylene (Fig. 5, E and F). In line 1 of *etr1-1(1–349)*, hypocotyl length decreased from 9.6 to 5.7 mm. In line 2 of *etr1-1(1–349)*, hypocotyl length decreased from 10.5 to 7.0 mm. In the control untransformed *etr1-7* line, hypocotyl length decreased from 9.7 to 1.8 mm. Thus, line 1 had a maximum ethylene response of 49% and line 2 had a maximum ethylene response of 44% compared with the control. The partial ethylene responsiveness of the *etr1-1(1–349)* transgenic lines does not result from a shift in ethylene sensitivity because the seedlings showed no significant change in hypocotyl length from 1 to 1,000 μL L^{-1} ethylene. In contrast to what we observed in the *etr1-7* background, two wild-type lines transformed with *etr1-1(1–349)* transgene showed no responsiveness to ethylene over all ethylene concentrations tested (Fig. 5, G and H).

Western-Blot Analysis of Transgenic Lines

We performed western-blot analysis to gain information on expression of the transgenes at the protein level. Western-blot analysis was performed on membranes isolated from the same transgenic lines used for the quantitative seedling growth response assay. The full-length receptor migrated at a molecular mass of 77 kD in the presence of reducing agent, consistent with the predicted molecular mass of 82 kD, and was recognized by both the anti-ETR1(165–400) and the anti-ETR1(401–738) antibodies (Fig. 6A). As expected, full-length receptor was detected in the wild-type and *etr1-1* backgrounds, but not in the *etr1-7* background. In addition, full-length receptor was detected in the *etr1-7* background when etr1-1 and etr1-1(G2) were transgenically expressed. Analysis with the anti-ETR1(401–738) antibody confirms

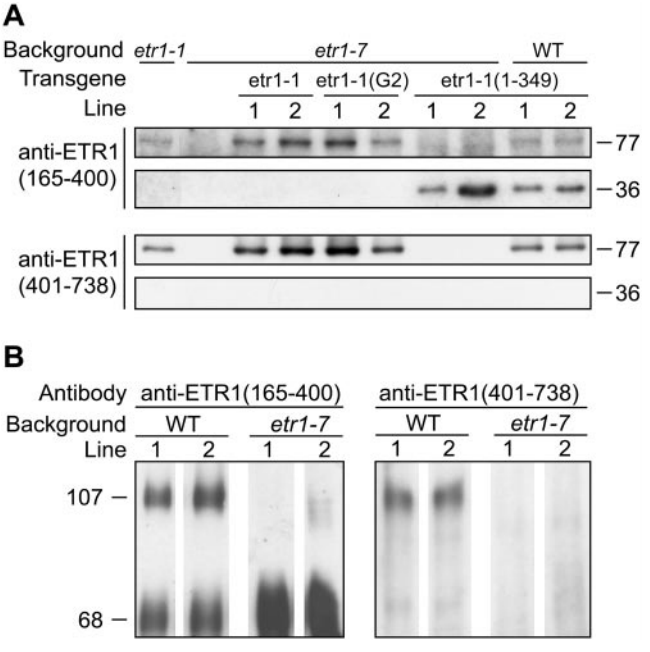

Figure 6. Western-blot analysis of transgenic lines containing mutant versions of etr1-1. Membrane fractions from etiolated Arabidopsis seedlings were incubated in the presence (A) or absence (B) of 100 mM dithiothreitol (DTT) for 1 h at 37°C. Protein (5 μg) was subjected to SDS-PAGE and then analyzed by western blot using the anti-ETR1(165–400) and anti-ETR1(401–738) antibodies. A, Expression levels of wild-type and mutant versions of ETR1 in different genetic backgrounds. In the presence of the reducing agent DTT, the full-length receptor migrates as a 77-kD monomer and the truncated etr1-1(1–349) receptor migrates as a 36-kD monomer. B, Disulfide-linked dimers formed by etr1-1(1–349) expressed in wild-type and *etr1-7* backgrounds. Positions of the 68-kD etr1-1(1–349) homodimer and of the putative 107-kD etr1-1(1–349):ETR1 dimer are indicated.

that no detectable full-length receptor is present in the *etr1-7* background line when expressing the truncated etr1-1(1–349) receptor. It should be noted that the anti-ETR1(165–400) antibody, but not the anti-ETR1(401–738) antibody, detects a minor protein migrating at slightly lower molecular mass than the 77-kD full-length receptor when etr1-1(1–349) is expressed in the *etr1-7* background. However, as discussed below, this protein is coincident with the 68-kD disulfide-linked dimer of etr1-1(1–349), and thus apparently represents residual protein that has not been completely reduced. The truncated ethylene receptor etr1-1(1–349) migrated at a molecular mass of 36 kD in the presence of reducing agent, consistent with the predicted molecular mass of 40 kD (Fig. 6A). The truncated ethylene receptor was recognized by the anti-ETR1(165–400) antibody but not by the anti-ETR1(401–738) antibody.

Differences in protein expression levels would represent a trivial explanation for the differences in the effectiveness of the various mutant forms of etr1-1 at conferring dominant ethylene insensitivity. However, the greater effectiveness of etr1-1 and etr1-1(G2) compared with etr1-1(1–349) in the *etr1-7* background did not correlate with higher levels of expression. For example, line 2 of etr1-1(G2) has a lower level of expression than either of the etr1-1(1–349) lines. These results are indicative that the C-terminal half of the protein, but not necessarily enzymatic activity, is required for maximal effectiveness of the etr1-1 mutant. In addition, the greater effectiveness of etr1-1(1–349) in the wild-type background compared with its effectiveness in the *etr1-7* background did not correlate with higher levels of expression in the wild-type background. For example, the highest level of etr1-1(1–349) was found in line 2 in the *etr1-7* background. These results are suggestive that the effectiveness of etr1-1(1–349) is greater in the presence of a full-length wild-type ETR1 receptor, potentially indicating some form of interaction between the two.

The ethylene receptor ETR1 has been demonstrated to form a disulfide-linked dimer, mediated by Cys-4 and Cys-6, that has an apparent molecular mass of 147 kD when analyzed by SDS-PAGE (Schaller et al., 1995). To assess whether the truncated etr1-1 receptor was still capable of dimerizing, we ran SDS-PAGE in the absence of reducing agent to preserve disulfide linkages (Fig. 6B). When visualized with anti-ETR1(165–400), a species with an apparent molecular mass of 68 kD was observed in both wild-type and *etr1-7* backgrounds. This oxidized species is approximately twice the mass of the 36-kD etr1-1(1–349) monomer and is not recognized by the anti-ETR1(401–738) antibody, consistent with the species representing a disulfide-linked dimer of the truncated receptor. It is interesting that a second major immunodetectable species of 107 kD was observed in the wild-type background, but was absent from the *etr1-7* background. The species of 107 kD is of a molecular mass consistent with that of a heterodimer between etr1-1(1–349) and the native wild-type ETR1. The presence of the native ETR1 at this molecular mass was confirmed by western blot with anti-ETR1(401–738), an antibody capable of recognizing the native full-length protein but not the truncated etr1-1 protein.

DISCUSSION

Genetic analysis supports the model shown in Figure 7, whereby ethylene receptors actively repress ethylene responses in the air (Fig. 7A; Hua and Meyerowitz, 1998; Bleecker, 1999). In the presence of ethylene, wild-type receptors switch to a signaling-inactive state that allows for induction of ethylene responses (Fig. 7B). Although His kinase activity has been demonstrated for the ethylene receptor ETR1 (Gamble et al., 1998), the role of this activity in signaling is unknown. Simple mutational analysis to uncover the function of the His kinase domain is confounded by the presence of other nonmutant members of the receptor family. Loss-of-function mutations in individual members of the receptor family have minimal effect upon the ability of Ara-

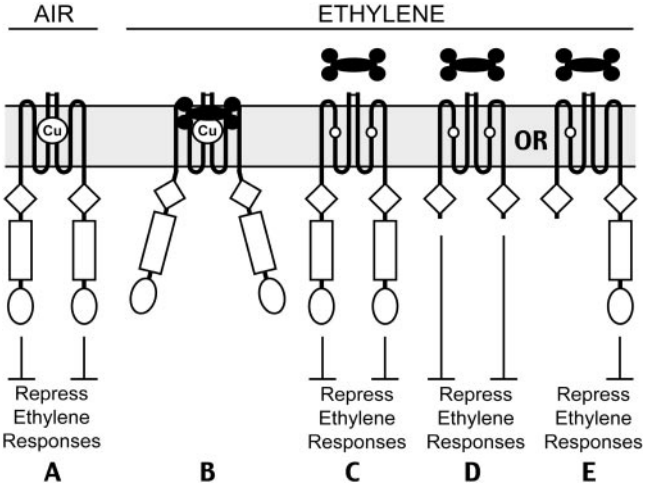

Figure 7. Models for signaling by wild-type and mutant versions of the ethylene receptor ETR1. The ethylene receptor ETR1 contains one ethylene-binding site per homo-dimer, with ethylene binding mediated by a single copper ion (Cu) present in the ethylene binding site. In air, receptors actively repress ethylene responses (A). In ethylene, receptors are inactivated, thereby relieving repression of the ethylene response pathway (B). The etr1-1 mutation (indicated by a white circle) eliminates binding of the necessary copper cofactor so that the receptor represses ethylene responses even in the presence of ethylene (C). A truncated etr1-1(1–349) receptor is still able to repress the ethylene responses. This could arise because of innate signaling activity of the etr1-1(1–349) receptor (D). Alternatively, etr1-1(1–349) could "convert" a full-length wild-type receptor to a ethylene-insensitive signaling state (E). A dimer between the truncated etr1-1(1–349) receptor and a full-length wild-type ETR1 receptor does not bind ethylene and consequently the ETR1 portion of the dimer represses ethylene responses.

bidopsis to respond to ethylene (Hua and Meyerowitz, 1998). We found that a G2 box mutation and a truncation of ETR1 had no apparent effect on the ability of the plant to respond to ethylene. However, ethylene responses could still be mediated by another member of the receptor family, such as ERS1, which contains a His kinase domain with all the conserved residues required for activity (Hua et al., 1995).

The problems inherent in mutational analysis of a gene family can be circumvented in part by use of gain-of-function mutations such as *etr1-1*. The etr1-1 mutant receptor of Arabidopsis is apparently locked into the signaling state that it has in the absence of ethylene. This state arises as a consequence of the mutant receptor's inability to bind ethylene, so that it represses ethylene responses even in the presence of ethylene (Fig. 7C; Schaller and Bleecker, 1995). Because etr1-1 remains locked in its active state in the presence of ethylene, when other members of the receptor family are signaling inactive, mutational analysis of etr1-1 can lend insight into the requirements for signaling by the receptor. Our focus in this set of experiments has been on etr1-1 and the role of the His kinase domain in signaling.

Our data indicate that enzymatic activity is not required for the ethylene insensitivity conferred by the mutant etr1-1 receptor. We found that an etr1-1 mutant that contains a G2 box mutation was as effective as etr1-1 itself in conferring ethylene insensitivity. As demonstrated in this report, the G2-box mutation eliminates His kinase activity in ETR1; this mutation is also predicted to eliminate any potential phosphatase activity of the protein (Yang and Inouye, 1993). It has been reported previously that etr1-1 is still able to confer dominant ethylene insensitivity when the His and Asp residues predicted to serve as phosphorylation sites are mutated (Chang and Meyerowitz, 1995). These data are consistent with the finding that ethylene-insensitive mutants can be generated in the ethylene receptors ETR2, ERS2, and EIN4, all of which contain diverged His kinase domains predicted to lack His kinase activity (Hua et al., 1998; Sakai et al., 1998). In addition, a tomato ethylene receptor with diverged kinase domain appears able to functionally compensate for an ethylene receptor containing a conserved kinase domain (Tieman et al., 2000).

We also found that ethylene insensitivity could be conferred by the truncated etr1-1(1–349) receptor that lacks the C-terminal half of the protein containing the His kinase domain. The truncated etr1-1(1–349) receptor conferred complete dominant ethylene insensitivity upon wild-type seedlings. However, etr1-1(1–349) did not confer ethylene insensitivity as effectively as full-length versions of etr1-1, when analyzed in the *etr1-7* genetic background that lacks ETR1. Dose response curves indicate that the ethylene responsiveness of these seedlings was reduced, rather than the sensitivity of the seedlings to ethylene shifted such that higher levels of ethylene were required to initiate the response. Partial ethylene insensitivity with a similar response to ethylene treatment has been observed in gene dosage experiments in which the ratio of wild-type to mutant *etr1-1* genes was increased by use of a triploid background (Hall et al., 1999). Partial ethylene insensitivity has also sometimes been observed when full-length *etr1-1* is expressed as a transgene in a wild-type background, presumably because of low expression of the transgene (Chang et al., 1993).

Use of the *etr1-7* background allowed us to directly compare protein expression levels from the various transgenes encoding full-length and truncated receptors. The decreased effectiveness of etr1-1(1–349) in the *etr1-7* background did not correlate with a reduced protein level compared with the full-length versions of etr1-1. Thus, our analysis of the truncated receptor supports a role for the C-terminal half of etr1-1 in the ability to confer ethylene insensitivity. The role of the C-terminal half for ethylene insensitivity could be in signal output, potentially for interactions with downstream signaling factors such as CTR1 (Kieber et al., 1993; Clark et al., 1998) because we found no evidence that His kinase activity was required for ethylene insensitivity. Alternatively, the C-terminal half could be important for proper folding of the protein; in such cases, the protein levels for etr1-1(1–349) determined by western-blot analysis may not accurately reflect the level of functional protein.

It is surprising that although the C-terminal half of etr1-1 increased the ability of etr1-1 to confer ethylene insensitivity under some conditions, it was not essential to this ability. Thus, the truncated version of etr1-1 is able to still mediate the active repression of ethylene responses. One explanation for this ability would be that the etr1-1(1–349) receptor is directly capable of signal output (Fig. 7D), potentially through its GAF domain (Aravind and Ponting, 1997), the function of which has not been determined for the ethylene receptors. Alternatively, the etr1-1(1–349) receptor might be incapable of signal output itself, but be able to "convert" other wild-type receptors to an ethylene-insensitive signaling state (Fig. 7E). One method by which such conversion could occur is suggested by our evidence that etr1-1(1–349) can form a disulfide-linked dimer with wild-type ETR1. Studies by Rodriguez et al. (1999) support the existence of a single ethylene-binding site per ETR1 dimer. As a consequence, a dimer of etr1-1(1–349) and ETR1 would likely result in neither polypeptide being able to bind ethylene because of the mutation within the shared ethylene-binding domain (Fig. 7E). The ETR1 portion of an etr1-1(1–349):ETR1 dimer thus would remain in a signaling-active state and still repress ethylene responses in the presence of ethylene. Our finding that the truncated etr1-1(1–349) re-

ceptor appeared to be more effective at conferring ethylene insensitivity when analyzed in a genetic background that still had a full-length wild-type ETR1 receptor is suggestive that interactions between etr1-1(1–349) and wild-type ETR1 may be of physiological relevance.

Dimerization of etr1-1(1–349) with ETR1 represents a mechanism by which ETR1 could be converted to an ethylene-insensitive signaling state. Whether etr1-1(1–349) is capable of converting other members of the ethylene receptor family besides ETR1 to an ethylene-insensitive signaling state remains an open question. In the *etr1-7* background, etr1-1(1–349) predominantly formed disulfide-linked homodimers, even though four non-ETR1 members of the Arabidopsis ethylene receptor family were present. However, different members of the ethylene receptor family could potentially interact through non-covalent associations. In bacteria, His kinases and the related chemoreceptors form non-covalently linked dimers that are important in signal propagation (Pan et al., 1993; Parkinson, 1993). In addition, bacterial chemoreceptors are hypothesized to propagate signals through noncovalent associations with neighboring receptors as large multimeric complexes (Bray et al., 1998). The discovery from crystal structure that the receiver domains of ETR1 form non-covalently linked dimers is indicative that noncovalent associations between ethylene receptors may play an important role in signaling (Muller-Dieckmann et al., 1999).

MATERIALS AND METHODS

Expression in Yeast (*Saccharomyces cerevisiae*)

For expression of GST fusions in yeast, the vector pEG(KT) was used (Mitchell et al., 1993). This vector contains the GST domain under control of a Gal-inducible promoter and allows for uracil selection in yeast. The GST fusions were designed to express that portion of ETR1 corresponding to amino acids 164 through 738 (Gamble et al., 1998). Site-directed mutation of ETR1 was performed using the Altered Sites Mutagenesis System (Promega, Madison, WI) according to the manufacturer and confirmed by sequencing. The G2-box mutation coded for a conversion of Gly-545 (GGG) to Ala (GCG) and a conversion of Gly-547 (GGC) to Ala (GCC). Transformation of yeast, isolation of GST fusion proteins, and in vitro analysis of His kinase activity was performed as previously described (Gamble et al., 1998). Upon request, these constructs and all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

Expression in Arabidopsis

For expression of *ETR1* in Arabidopsis, a 7.3-kb genomic clone containing promoter, coding, and downstream sequence (Chang et al., 1993) was ligated into the *Bam*HI and *Sal*I sites of the binary vector pBIN19 (Bevan, 1984) as previously described (Hall et al., 1999). For expression of *etr1-1*, a 7.3-kb genomic clone with *Bam*HI linkers (Chang et al., 1993) was ligated into pBIN19. For construction of site-directed mutations, the *ETR1* and *etr1-1* genomic clones were subcloned into pALTERII and mutations made using the Altered Sites Mutagenesis System (Promega). To produce truncated versions of the receptor, site-directed mutations were introduced that converted the codon for Met-350 (ATG) into a Stop codon (TAG). Site-directed mutants were confirmed by sequencing, excised from the pALTERII vector, and subcloned into pBIN19.

Constructs in the pBIN19 vector were introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform Arabidopsis ecotype Columbia by the dipping method (Bent and Clough, 1998). Seeds were plated onto agar plates, and transformed plants selected based on kan^r (50 μg mL^{-1}). Plants were allowed to self-pollinate and homozygous lines identified in subsequent generations. Arabidopsis plants were grown in a 3:1 (v/v) mixture of Metromix 360 (Scotts-Sierra Horticultural Products, Marysville, OH) to perlite, and watered with 10% (v/v) Hoagland solution. Plants were maintained in an environmental growth chamber at 22°C with an 18-h daylength.

PCR was used to confirm the presence of the G2 mutation in the etr1-1(G2) transgenic line (*etr1-7* background). Seedling tissue was alkali treated and PCR performed as described (Klimyuk et al., 1993) using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Amplification was performed using 5′ primer ATGCTCATGATCTGTCTACGCTACG and 3′ primer TTACCCTCCATCAGATTCACAAACC. The PCR product was cloned into the vector pstBlue-1 according to the manufacturer (Novagen, Madison, WI), and the region encoding the G2 box sequenced.

Seedling Growth Response Assays

To examine the triple response of seedlings to ethylene (Chen and Bleecker, 1995; Hall et al., 1999), seeds were plated on petri dishes containing one-half-strength Murashige and Skoog basal media with Gamborg's vitamins (Sigma, St. Louis) and 0.8% (w/v) agar. Aminoethylvinyl-Gly (5 μM) was included in the growth media to inhibit ethylene biosynthesis by the seedlings. Plates were placed at 4°C in the dark for 2 d to help coordinate seed germination, and then placed at 22°C under fluorescent light for 8 h. Plates were then placed in 4-L containers and seedlings grown in the dark. For the experiments shown in Figures 3 and 4, flow-through containers were used with an ethylene concentration of 35 μL L^{-1}. For the experiment shown in Figure 5, ethylene was added to sealed containers at the desired concentration. Seedlings were examined after 4 d, time 0 corresponding to when the plates were removed from 4°C and brought to 22°C. To measure hypocotyl length, seedlings were grown on vertically oriented plates. Seedlings on the plates were scanned using Photoshop (Adobe Systems, Mountain View, CA) and a LaCie scanner, and measurements made using NIH Image (version 1.60, National Institutes of Health, Bethesda, MD).

Membrane Protein Isolation

For isolation of Arabidopsis membranes, 4-d-old etiolated seedlings (1 g) were homogenized at 4°C in extraction buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 10 mM EDTA; and 20%[v/v] glycerol) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg mL^{-1} leupeptin, and 1 μg mL^{-1} pepstatin as protease inhibitors. The homogenate was strained through Miracloth (Calbiochem-Novabiochem, San Diego) and centrifuged at 8,000*g* for 15 min. The supernatant was centrifuged at 100,000*g* for 30 min, and the membrane pellet resuspended in 10 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; and 10% (v/v) glycerol with protease inhibitors. Protein concentration was determined by a modification of the Lowry method, in which membrane proteins were extracted with 0.4% (w/v) deoxycholate (Schaller and DeWitt, 1995). Bovine serum albumin was used as the protein standard.

Western-Blot Analysis

Proteins were resuspended in SDS/PAGE loading buffer in the absence or presence of 100 mM DTT (Schaller et al., 1995). The reductant DTT was left out of the loading buffer when it was desired to preserve the disulfide-linked dimer of ETR1 (Schaller et al., 1995). Membrane proteins were treated at 37°C for 1 h and then fractionated by SDS-PAGE on either 8% or 10% (w/v) polyacrylamide gels (Laemmli, 1970). After electrophoresis, proteins were electroblotted to Immobilon nylon membrane (Millipore, Bedford, MA). Two antibodies were used for western-blot analysis. One antibody, termed anti-ETR1(401–738), was generated against amino acids 401 through 738 of ETR1 (Schaller et al., 1995), and was used to identify versions of ETR1 that contained the His kinase domain. A second antibody, termed anti-ETR1(165–400), was generated against amino acids 165 through 400 of ETR1 (Schaller et al., 1995), and was used to identify truncated versions of ETR1 that lacked the His kinase domain. The anti-ETR1(165–400) antibody recognizes the dimeric form of ETR1 preferentially over the monomeric form (Schaller et al., 1995) so, unless indicated otherwise, analysis with the anti-ETR1(165–400) antibody was performed on proteins that were not treated with DTT. Immunodecorated proteins were visualized by enhanced chemiluminescence detection according to the manufacturer (Pierce Chemical, Rockford, IL).

For the results shown in Figure 6, the polyclonal anti-ETR1(165–400) serum was affinity purified. This was done to remove antibodies that cross-reacted with Arabidopsis polypeptides of similar molecular mass to the reduced forms of the full-length and truncated receptors. The anti-ETR1(165–400) serum was depleted of antibodies that cross-react with GST by passing through a column of Affigel-10 cross-linked to GST. The serum was then affinity purified with Affigel-10 columns cross-linked to GST-ETR1(165–400) (Schaller et al., 1995). Antibodies were eluted with 100 mM Gly, pH 2.5, neutralized with 1 M Tris, pH 8.0, and dialyzed against phosphate-buffered saline. Cross-linking to Affigel-10 was performed according to the manufacturer (Bio-Rad Laboratories, Hercules, CA).

ACKNOWLEDGMENTS

We thank Estelle Hrabak and John Collins for critical reading of the manuscript before publication.

Received August 23, 2001; returned for revision November 16, 2001; accepted January 11, 2002.

LITERATURE CITED

Abeles FB, Morgan PW, Saltveit ME Jr (1992) Ethylene in Plant Biology, Ed 2. Academic Press, San Diego

Aravind L, Ponting CP (1997) The GAF domain: an evolutionary link between diverse phototransducing proteins. Trends Biochem Sci **22:** 458–459

Bent AF, Clough SJ (1998) Agrobacterium germ-line transformation: transformation of Arabidopsis without tissue culture. *In* SB Gelvin, RA Schilperout, eds, Plant Molecular Biology Manual, Ed 2. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1–14

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res **12:** 8711–8721

Bilwes AM, Alex LA, Crane BR, Simon MI (1998) Structure of CheA, a signal-transducing histidine kinase. Cell **96:** 131–141

Bleecker AB (1999) Ethylene perception and signaling: an evolutionary perspective. Trends Plant Sci **4:** 269–274

Bleecker AB, Estelle MA, Somerville C, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science **241:** 1086–1089

Bray D, Levin MD, Morton-Firth CJ (1998) Receptor clustering as a cellular mechanism to control sensitivity. Nature **393:** 85–88

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene response gene *ETR1*: Similarity of product to two-component regulators. Science **262:** 539–544

Chang C, Meyerowitz EM (1995) The ethylene hormone response in Arabidopsis: a eukaryotic two component signaling system. Proc Natl Acad Sci USA **92:** 4129–4133

Chang C, Shockey JA (1999) The ethylene-response pathway: signal perception to gene regulation. Curr Opin Plant Biol **2:** 352–358

Chen QG, Bleecker AB (1995) Analysis of ethylene signal transduction kinetics associated with seedling-growth responses and chitinase induction in wild-type and mutant Arabidopsis. Plant Physiol **108:** 597–607

Clark KL, Larsen PB, Wang X, Chang C (1998) Association of the Arabidopsis CTR1 Raf-like kinase with the ETR1 and ERS1 ethylene receptors. Proc Natl Acad Sci USA **95:** 5401–5406

Duclos B, Marcandier S, Cozzone AJ (1991) Chemical properties and separation of phosphoamino acids by thin-layer chromatography and/or electrophoresis. Methods Enzymol **201:** 10–21

Gamble RL, Coonfield ML, Schaller GE (1998) Histidine kinase activity of the ETR1 ethylene receptor from Arabidopsis. Proc Natl Acad Sci USA **95:** 7825–7829

Guzmán P, Ecker JR (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2:** 513–523

Hall AE, Chen QG, Findell JL, Schaller GE, Bleecker AB (1999) The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the ETR1 ethylene receptor. Plant Physiol **121:** 291–299

Hua J, Chang C, Sun Q, Meyerowitz EM (1995) Ethylene sensitivity conferred by Arabidopsis *ERS* gene. Science **269:** 1712–1714

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell **94:** 261–271

Hua J, Sakai H, Nourizadeh S, Chen QG, Bleecker AB, Ecker JR, Meyerowitz EM (1998) EIN4 and ERS2 are members of the putative ethylene receptor family in Arabidopsis. Plant Cell **10:** 1321–1332

Kieber JJ, Rothenberg M, Roman G, Feldman KA, Ecker JR (1993) *CTR1,* a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. Cell **72:** 427–441

Klimyuk VI, Carroll BJ, Thomas CM, Jones JDG (1993) Alkali treatment for the rapid preparation of plant material for reliable PCR analysis. Plant J **3:** 493–494

Knight LI, Rose RC, Crocker W (1910) Effects of various gases and vapors upon etiolated seedlings of the sweet pea. Science **31:** 635–636

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Mitchell DA, Marshall TK, Deschenes RJ (1993) Vectors for inducible overexpression of glutathione *S*-transferase fusion proteins in yeast. Yeast **9:** 715–723

Muller-Dieckmann HJ, Grantz AA, Kim SH (1999) The structure of the signal receiver domain of the *Arabidopsis thaliana* ethylene receptor ETR1. Struct Fold Des **7:** 1547–1556

Pan SQ, Charles T, Jin S, Wu Z-L, Nester EW (1993) Preformed dimeric state of the sensor protein VirA is involved in plant-*Agrobacterium* signal transduction. Proc Natl Acad Sci USA **90:** 9939–9943

Parkinson JS (1993) Signal transduction schemes of bacteria. Cell **73:** 857–871

Rodriguez FI, Esch JJ, Hall AE, Binder BM, Schaller GE, Bleecker AB (1999) A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science **283:** 996–998

Sakai H, Hua J, Chen QG, Chang C, Medrano LJ, Bleecker AB, Meyerowitz EM (1998) *ETR2* is an *ETR1*-like gene involved in ethylene signaling in *Arabidopsis*. Proc Natl Acad Sci USA **95:** 5812–5817

Schaller GE (2000) Histidine kinases and the role of two-component systems in plants. Adv Bot Res **32:** 109–148

Schaller GE, Bleecker AB (1995) Ethylene-binding sites generated in yeast expressing the Arabidopsis *ETR1* gene. Science **270:** 1809–1811

Schaller GE, DeWitt ND (1995) Analysis of the H^+-ATPase and other proteins of the Arabidopsis plasma membrane. Methods Cell Biol **50:** 129–148

Schaller GE, Ladd AN, Lanahan MB, Spanbauer JM, Bleecker AB (1995) The ethylene response mediator ETR1 from Arabidopsis forms a disulfide-linked dimer. J Biol Chem **270:** 12526–12530

Stock JB, Surette MG, Levit M, Park P (1995) Two-component signal transduction systems: structure-function relationships and mechanisms of catalysis. *In* JA Hoch, TJ Silhavy, eds, Two-Component Signal Transduction. American Society for Microbiology, Washington, DC, pp 25–51

Tieman DM, Klee HJ (1999) Differential expression of two novel members of the tomato ethylene-receptor family. Plant Physiol **120:** 165–172

Tieman DM, Taylor MG, Ciardi JA, Klee HJ (2000) The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. Proc Natl Acad Sci USA **97:** 5663–5668

van Hoof A, Green PJ (1996) Premature nonsense codons decrease the stability of phytohemagglutinin mRNA in a position-dependent manner. Plant J **10:** 415–424

Wilkinson JQ, Lanahan MB, Clark DG, Bleecker AB, Chang C, Meyerowitz EM, Klee HJ (1997) A dominant mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants. Nature Biotechnol **15:** 444–447

Yang Y, Inouye M (1993) Requirement of both kinase and phosphatase activities of an *E. coli* receptor (Taz1) for ligand-dependent signal transduction. J Mol Biol **231:** 335–342